PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/10, 15/79, A61K 48/00 — A1

(11) International Publication Number: WO 92/21750

(43) International Publication Date: 10 December 1992 (10.12.92)

(21) International Application Number: PCT/US92/04011

(22) International Filing Date: 21 May 1992 (21.05.92)

(30) Priority data:
707,055 29 May 1991 (29.05.91) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent). IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: EUKARYOTIC EXPRESSION VECTORS WITH REGULATION OF RNA PROCESSING

(57) Abstract

The present invention relates to methods of increasing the efficiency of foreign gene expression in cells. In particular, the present invention relates to the use of retroviral vector mediated gene transfer techniques and results in methods of increasing the viral titer and the amount of protein expressed. The method of the present invention and constructs used therein are suitable for use in human gene therapy, particularly for the treatment of AIDS.

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EUKARYOTIC EXPRESSION VECTORS WITH REGULATION OF RNA PROCESSING

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods of increasing the efficiency of foreign gene expression in target cells. The present invention further relates to host cells and constructs for use in such methods.

Background of the Invention

Retroviruses are being used with increased frequency as vectors to introduce foreign genes into eukaryotic cells. These retroviral vectors utilize the ability of retroviruses to transfer genes with high efficiency into a wide variety of cell types and provide a tool for the expression of foreign genes in the target cell. This technique can be used to introduce genes into human somatic cells (Williams, Human Gene Therapy 7:229-239; 1990) and has application to human gene therapy.

For example, retroviral vectors have been used to transduce hematopoietic cells that might be used for gene therapy of diseases affecting blood cells. However, progress in obtaining stable and long term expression of the introduced genes has been slow, frustrating and only partially successful. This is due, in part, to a lack of sufficient understanding of the control of gene expression in eukaryotic cells.

Retroviral vectors contain cis-acting viral sequences necessary for the viral life cycle. These include the packaging sequences (ψ), reverse transcription signals, integration signals and viral

promoter enhancer and polyadenylation sequences. CDNAs, minigenes and whole genes can be inserted into the retroviral vector. The inserted gene can be expressed from the viral transcriptional regulatory sequences and/or from additional foreign promoter elements in the vector (for example, CEM or SV40).

The retroviral vectors must be introduced into a "packaging-line" to make virus particles. These lines are usually derived from mouse fibroblasts and contain all of the structural protein expressed that is necessary for the production of retrovirus particles. However, these lines do not contain the packaging signal (ψ). Thus, after introduction of the retroviral vector (containing ψ), the vector RNA will be packaged into viral particles.

The use of retroviral vectors for the efficient transfer of genes has been hampered by two major problems. The first is obtaining efficient expression of the retrovirally carried gene in the transduced cell. Two techniques have been described which improve gene expression. The first method involves a self inactivated (SIN) vector. In this vector, the LTR-driven gene expression is eliminated in the infected cells, relieving interference with the expression of the transduced gene [Yu et al, PNAS 83: 3194-3198; 1986]. The use of the SIN vectors, however, has been limited by low virus titer.

The second technique, that of Hantzopoulos et al [PNAS 86: 3519-3523; 1989], is based on the insertion of the foreign gene in the retrovirus

vector outside of the transcriptional unit. This vector, called double-copy (DC), is designed to generate a transcript without the interference of a read-through transcript initiated from the viral LTR.

The second major problem involved in the efficient transfer of genes is the generation of high viral titers in the host cell. High virus titers result in improved efficiency of gene transfer in vitro and in vivo. Since retroviruses lose infectivity during concentration, high efficiency gene transfer can only be achieved with high titered virus.

The present invention overcomes the problems of inefficient gene expression and low viral titers. The method of the present invention increases the titer and the efficiency of foreign gene expression by utilizing the Rev and Rev-responsive-element (RRE) of primate lentiviruses.

Primate lentiviruses, such as HIV, SIV and HTLV, regulate processing and transport of RNA in two ways. First, these viruses express small regulatory proteins from mRNAs which are processed by multiply splicing. In addition, structural proteins are expressed from unspliced and single spliced RNA by these viruses are transported by a regulatory protein (rev or rex) which recognizes an RRE RNA element within the mRNA. The regulatory protein together with cellular factors, transports RRE containing RNAs from the nucleus to the cytoplasm of the cell where the RNA is translated. In the absence of the regulatory protein (rev or

rex), unspliced and single spliced messages are not detected in the cytoplasm. (HTLV-I rex can functionally replace HIV-1 and HIV-2 rev, see Sakai et al, J. Virology 64: 5833-5839; 1990). Utilizing the primate lentivirus transport system, the present invention provides for the efficient transport of foreign genes.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide for efficient gene expression in eukaryotic cells.

It is another object of the present invention to provide a retroviral vector for use in gene therapy.

Various other objects and advantages of the present will be apparent from the following description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a retroviral vector of the present invention which contains RRE or RRE within an intron of the foreign gene.

Figure 2 depicts a retroviral vector of the present invention showing possible positions of the RRE or RRE-intron in the vector.

Figure 3 depicts a retroviral vector of the present invention showing the use of internal promoters in the transcription the foreign gene.

Figure 4 depicts the regulation of the protective gene expression.

DETAILED DESCRIPTION OF THE INVENTION

The present invention addresses the shortcomings found when existing vectors are used to transfer genes to eukaryotic cells. In particular, the present invention relates to a method which increases the viral titer and the efficiency of protein expression, and to vectors suitable for use in same. These advantages are accomplished by utilizing the processing and transport mechanism of primate lentiviruses.

Viral titer depends on three factors: 1) the amount of RNA transcripts made from the vector long terminal repeat (LTR), 2) the amount of structural protein expressed in the cell, and 3) the amount of packagable RNA transported from the nucleus to the cytoplasm. The method of the present invention increases the amount of packagable RNA in the cytoplasm and thereby the viral titer by incorporating a transport protein responsive element (for example, RRE or RxRE) into the RNA to be RRE transported, in a cell producing a transport protein (for example, revI, revII or rex).

The present invention also increases the amount of protein expressed in the host cell and target cell. The amount of protein expressed depends somewhat on the regulation of the processing and transport of the mRNA. It has been estimated that only about one-twentieth of the total mass of hnRNA made ever leaves the nucleus. It seems,

therefore, that a portion of the primary transcripts is degraded in the nucleus even if they contain the natural processing signals. Studies on mutant yeast suggest that transport of RNA containing a splice site can occur only if the splicing is completed.

As most eukaryotic RNA is processed through splicing, a majority of the RNA transcribed from a eukaryotic expression vector contains an intron and will be spliced in the cytoplasm of the host cell. Most of the known retroviral vector constructs do not contain introns, as introns cannot be packaged with high efficiency into viral particles. However, to achieve high level gene expression in the target cells, a gene must contain processing signals (for example, splice signals). If intron containing RNA cannot be packaged into the viral particles, high level gene expression after retroviral mediated gene transfer will not be achieved. Correct processing is very important for high efficiency foreign gene expression using retroviral vectors.

In the present invention intron-containing RNA is transported and packaged by the host cell. This is achieved by incorporating a processing signal element, (for example, RRE) in the intron-containing RNA and expressing the RNA in a cell producing the processor protein (for example, rev or rex). A retroviral processing signal is an RNA element which is responsive to retroviral processing factors. For example, RRE is responsive to rev and rex while RxRE is responsive to rex (HTLV-I). RREπ (π= two) is responsive to revπ (two). These

elements function similarly processing the retroviral RNAs by the same mechanism.

When such RNA is expressed in the nucleus, the rev or rex, together with cellular factors, transports the intron-containing RNA into the cytoplasm where it is packaged. In target cells infected with the viral particles, the intron containing RNA can be incorporated into the genome and high level of gene expression can be achieved through splicing.

In the present invention, known retroviral regulatory elements are incorporated into different expression vectors (for example, eukaryotic expression vectors or retroviral vectors). These elements are known to function in human or monkey cells and may function in other cells, for example mouse cells. Thus, preferred packaging cell lines for use in the present invention are derived from monkey or human cells. Such packaging cell lines are established using methods known in the art.

Eukaryotic expression vectors of the present invention comprise a DNA sequence encoding a foreign gene which is operably linked to a sequence encoding an RNA element (processing signal elements) which is responsible for processing of the RNA by retroviral trans-acting elements like rev or rex (see Figure 1).

Vectors suitable for use in the present invention include eukaryotic expression vectors such as pMAMneo (Clonetech) and pSG5 (Stratagene). In a preferred embodiment, the vector is a retroviral vector such as DC-vector (E. Gilboa), pLX (D. Miller) and pG (GTI). One skilled in the art will

appreciate that the vectors to which the invention relates include strong promoters such as a long terminal repeat (LTR), CMV, TK or SV40 and other eukaryotic promoters. Preferred LTRs include the Moloney LTRs and HIV LTRs.

The retroviral vectors of the invention also include a packaging signal. The packaging signal functions to identify the retroviral vector transcripts for packaging into viral particles.

The choice of foreign genes to be inserted in the vector is dependent upon the effect sought to be achieved. Such genes include ß-globin and adenosine deaminase (ADA), with ADA being preferred. When treatment of AIDS infected patients is to be effected, the foreign gene can encode poly-TARs as described in the application Serial No. 07/596,299, the entire contents of which is hereby incorporated by reference. The foreign genes can be, advantageously, cDNA sequences.

In addition to the foregoing components, the vectors of the present invention can also include a sequence encoding for a selection marker, for example, a neomycin gene or a hygromycin gene.

The above components can be positioned in the vector such that the foreign gene is operably linked to the promoter. Further, the sequence encoding the processing element signal, such as RxRE, is operably linked to the foreign gene. One skilled in the art will appreciate that as the function of the processing signal elements such as RRE is orientation dependent, the RRE, or functional equivalent thereof, must be in the same orientation as the foreign gene transcript. The RRE or

functionally equivalent portion thereof must be located within the transcriptional unit of the foreign gene or within the transcriptional unit of the vector.

The processing signal element, for example, RRE is located within the vector so as not to disturb the packaging or transcriptional functions of the vector. For example, the RRE can be insert in the vector in the LTR, in front of the foreign gene, behind the foreign gene or within an intron of the foreign gene (see Figure 2).

The vector can also contain internal promoters operably linked to the foreign gene and the DNA sequence encoding the processing signal element (such as RRE) thereby producing a second transcript. The internal promoter can be in the same orientation as the vector promoter, for example, a 5' LTR, or in the opposite orientation (See Figure 3). Since the RRE is orientation dependent, when the internal promoter and the RRE is in the same orientation as the 5' LTR, than rev can transport the transcript from the 5' LTR as well as the second transcript. However, when the internal promoter and the RRE are in the opposite direction as the vector promoter, only the transcript coming from the internal promoter will be transported by rev.

In the method of the present invention, the vectors are introduced into a packaging host cell, for example, by transfection. Suitable packaging cells express a transport protein/processor protein (for example, revI, revII or rex). When retroviral vectors are used, the

packaging cells are such that all the retroviral proteins necessary for assemblage of viral particles are produced. Examples of suitable packaging cells include mice packaging cells, for example, PA317, monkey packaging cells, and human packaging cells.

Packaging host cells for use in the present invention can be generated which constitutively express the rev or rex protein using methods known in the art. For example, host cells which express rev or rex can be created by infecting the cell with a construct encoding and expressing the desired protein, preferably from a strong promoter, such as CMV or SV-40.

The foregoing steps of the present method are exemplified as follows. Vectors contemplated by the present invention are constructed using methods well known in the art. For example, a LXSN vector containing a neomycin gene expressed from an SV-40 promoter and a LASN vector which consists of the LXSN vector with an ADA gene inserted under the control of the viral LTR [Hock et al, Blood 74: 876-881; 1989] are constructed. In both vectors, the RRE is inserted downstream from the SV-40 promoter. The LXSN or LASN vectors can then be cotransfected with a rev gene-containing plasmid (such as, CMV-rev) into, for example, a PA317 amphotropic producer cell line if rex is functional in the murine cells. Otherwise, a human or monkey packaging line can be used. The cell line is transfected at a ratio of about 5:1 to 20:1 rev plasmid to retroviral vector to ensure the insertion of the rev gene in the cell where the retroviral vector is inserted. G418 resistant producer cells can then be isolated.

In retroviral-mediated gene transfer, target cells (for example, lymphocytes) are infected with the retroviral particles using common virus infection method. To enrich the foreign-gene containing cell population, the infected cells are then selected using the neomycin analog G418.

With other non-retroviral eukaryotic expression vectors, the target cells are transfected directly. However, direct transfection results in low efficiency gene transfer.

In the case of retroviral vectors, in the method of the present invention, the transfected vector integrates into the genome of the host cell. Transcripts are produced in the cell containing the RRE then transported from the nucleus to the cytoplasm with the help of rev or rex thereby increasing the amount of packagable RNA and thus the viral titer. The viral particles can be used to infect target animal or human cells, for example, in gene therapy treatments.

Target cells can be selected based on the treatment to be effected. In one embodiment, stem cells from bone marrow are used. For bone marrow stem cells gene transfer see, for example, Williams, D.A. (1970) Human Gene Therapy 1: 229-239. Alternatively, lymphocytes can be used as cellular vehicles for gene therapy [Culvert et al, (1991) PNAS 88: 3155-3159].

The method of the present invention can be used in human gene therapy to effect the expression of a foreign gene in a patient. Vectors of the present invention encoding a foreign gene to be expressed are transfected into a eukaryotic

packaging host cell expressing the necessary transport protein. Co-culturing these packaging cells with cells from the patient, preferably stem cells from the bone marrow, the sequence encoding the foreign gene is transferred to the patient cells where it is translated and the foreign gene expressed. Alternatively, bone marrow cells or lymphocytes can be directly infected with the retroviral particles when they are stimulated to divide. The patient cells containing the foreign gene are then separated from the host cells and administered back to the patient, for example, intravenously.

The method of the present invention can further be exemplified in the treatment of HIV infected patients. Vectors for use in the treatment of HIV patients can include a strong promoter which is switched on in the presence of the virus or viral-transactivator protein (tat), but in the absence of viral infection, the promoter does not express the product, preferably HIV LTRs (see Figure 4). Preferably, the foreign gene encodes poly TARs. The vectors are introduced into a packaging host cell expressing both a transport protein, for example, rev, and the tat protein.

For the treatment of AIDS, the protective gene which protects the cell against HIV replication should be expressed in the infected cells, but not in healthy non-HIV infected cells. Since HIV tat protein does not have a cellular analog, HIV-LTR can be used as a promoter in the eukaryotic vectors of the present invention. HIV-LTR has low background activity in the absence of tat, yet in the presence

of tat the promoter is trans-activated about 100-1000 fold. Using HIV-LTR for the treatment of AIDS ensures a biological regulation of the protective gene expression.

The protective gene can be any gene encoding a product which is able to dominantly interfere with the viral replication. Examples of such genes include, poly-TAR elements which inhibit tat function, ribozymes directed against gag mRNA protein [Sarver et al, (1990) Science 247: 1222-1225], and trans-dominant-mutants, such as a mutant gag protein which inhibits viral release from the cells [Trono et al, (1989) Cell 59: 113-120]. In the case of AIDS treatment, HIV-infected cells express the rev protein, which ensures the processing of the protective gene through RRE-rev interaction in the target cells.

Since the HIV-LTR is a strong promoter only in the presence of tat, the packaging cell line, used for the production of viral particles containing the protective gene, have to produce tat and rev. Thus, the packaging cell line must be derived from human cells (for example, HELA) or monkey cells (for example, COS).

* * * * *

All publications hereinabove are hereby incorporated by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the scope of the invention.

WHAT IS CLAIMED IS:

1. An eukaryotic expression vector comprising a strong promoter operably linked to a DNA sequence encoding a foreign gene and to a second DNA sequence encoding an RNA processing signal element.

2. The vector according to claim 1 which is a retroviral vector.

3. The vector according to claim 1 wherein said processing signal element is a retroviral processing signal element.1

4. The vector according to claim 3 wherein said element is a Rev responsive element (RRE) or a Rex responsive element (RxRE).

5. The vector according to claim 1 wherein said processing signal element is located within the transcriptional unit of the foreign gene.

6. The vector according to claim 2 wherein said processing signal element is located within the transcriptional unit of the retroviral vector.

7. The vector according to claim 1 further comprising a selectable marker.

8. The vector according to claim 1 further comprising an internal promoter operably linked to said foreign gene.

9. The vector according to claim 1 further comprising a DNA segment encoding a viral or cellular transactivator protein operably linked to said promoter.

10. The vector according to claim 9 wherein said transactivator protein is tat.

11. A eukaryotic host cell expressing a transport protein comprising the vector of claim 1.

12. The host cell according to claim 11 wherein the transport protein is rev or rex.

13. A method of expressing a foreign gene in a patient comprising the steps of:

i) culturing eukaryotic host cells of claim 11 with patient cells under conditions such that said sequence encoding said foreign gene is transferred from said host cells to said patient cells and is translated in said patient cells;

ii) separating said host cells and said patient cells;

iii) administering said patient cells to said patient.

14. The method according to claim 13 wherein said patient cells are stem cells from bone marrow.

15. The method according to claim 13 wherein said foreign gene is adenosine deaminase.

16. The method according to claim 13 wherein said foreign gene is a poly TAR.

FIG. 1

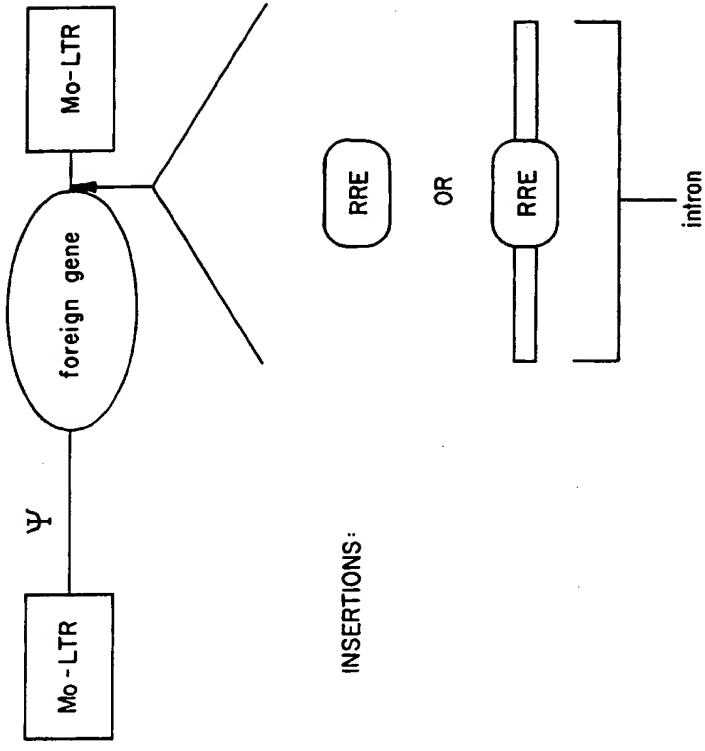


FIG. 2

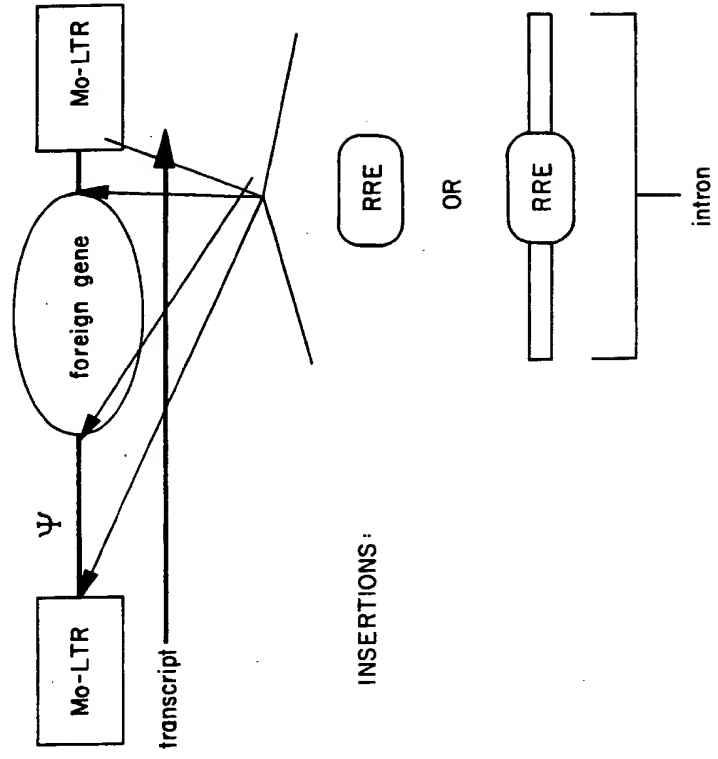
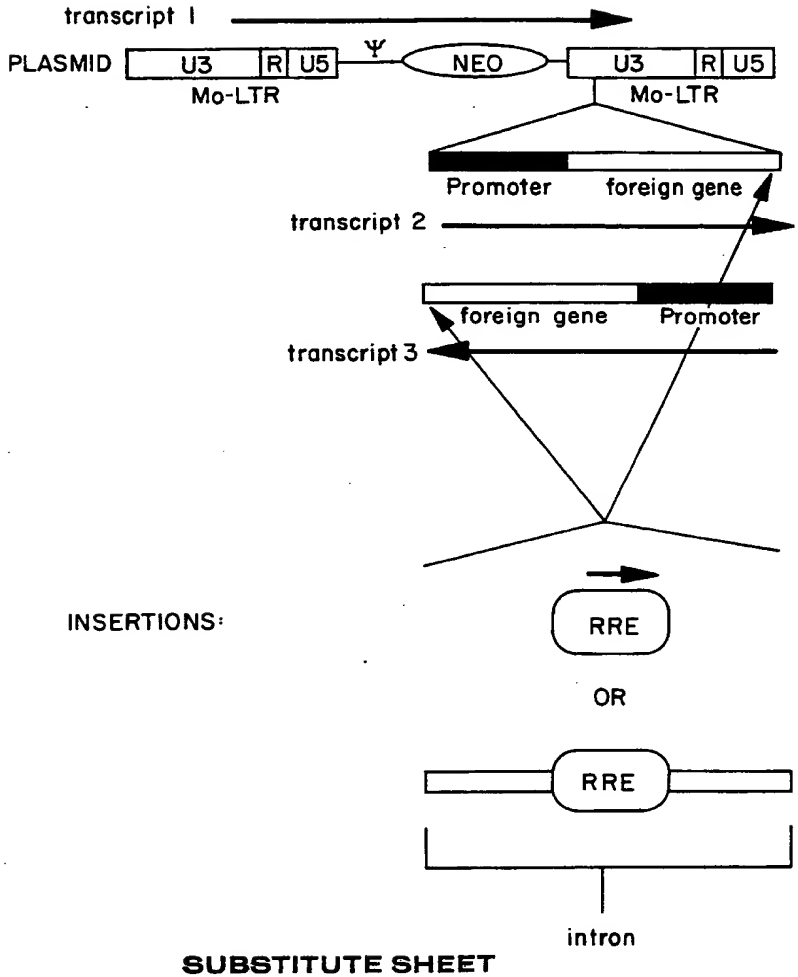

Mo-LTR
foreign gene
Ψ
Mo-LTR
transcript
INSERTIONS:
RRE
OR
RRE
intron

FIG. 3


transcript 1
PLASMID
U3
R
U5
Mo-LTR
Ψ
NEO
U3
R
U5
Mo-LTR
Promoter
foreign gene
transcript 2
foreign gene
Promoter
transcript 3
INSERTIONS:
RRE
OR
RRE
intron

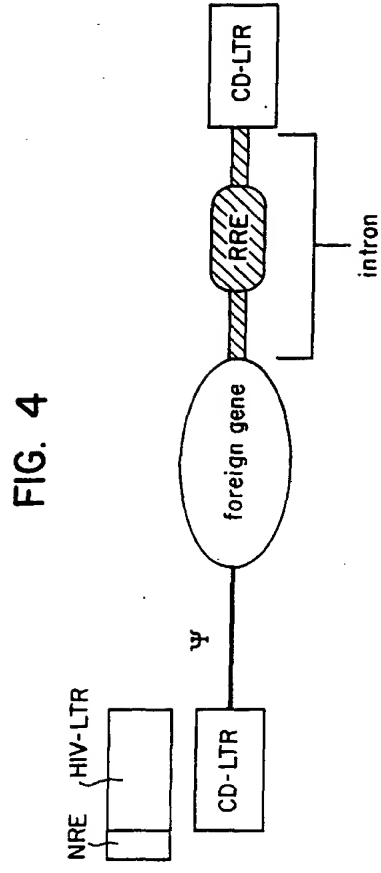


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04011

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/10, 15/79; A61K 48/00
US CL :435/240.1, 320.1; 424/93R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.1, 320.1; 424/93R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1985+, AUTOMATED PATENT SEARCH (U.S.)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Volume 64, Number 4, issued April 1990, N. Lewis et al, "Identification of a cis-Acting Element in Human Immunodeficiency Virus Type 2 (HIV-2) That Is Responsive to the HIV-1 rev and Human T-Cell Leukemia Virus Types I and II rex Proteins", pages 1690-1697, see entire article.	1-15
Y	Journal of Virology, Volume 64, No. 6, issued June 1990, H. Toyoshima et al, "Secondary Structure of the Human T-Cell Leukemia Virus Type 1 rex-Responsive Element Is Essential for rex Regulation of RNA Processing and Transport of Unspliced RNAs", pages 2825-2832, see entire document.	1-15
Y	Journal of Virology, Volume 64, Number 6, issued June 1990, A. J. Smith et al, "Human Immunodeficiency Virus Type 1 $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ Expressed from a Simian Virus 40 Late Replacement Vector Are Efficiently Processed and Assembled into Viruslike Particles", pages 2743-2750, see entire article.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search 26 August 1992	Date of mailing of the international search report 02 SEP 1992
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04011

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Science, Volume 247, issued 16 February 1990, H. S. Olsen et al, "Secondary Structure Is the Major Determinant for Interaction of HIV rev Protein with RNA", pages 845-848, see entire article and Figure 4, especially.	1-7 1-15
Y	Trends in Genetics, Volume 7, Number 1, issued January 1991, C. A. Rosen, "Regulation of HiV gene expression by RNA-protein interactions", pages 9-14, see entire article.	1-15